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14. ABSTRACT
We are studying the utility of using B.fragilis OmpA as a vehicle on which to put antigenic epitopes of organisms that can be used as bioterror agents, with the aim of eventually constructing a vaccine vehicle vector. OmpA is the major outer membrane protein of B. fragilis, a gram negative anaerobe that normally resides in the gut. There are four homologs for ompA in the genome. The purpose of this study was to construct a B. fragilisompA deletant and to begin to characterize the function of OmpA, as well as the specific function(s) of the various loops exposed on the surface. We used a PCR-based site-directed mutagenesis technique to insert the FLAG marker into recombinant ompA cloned in the pET-27b(+) expression system. Initial studies indicated difficultyin achievingstableexpression, export to periplasm and integration of B. fragilis OmpA into the E. coli membrane. Thus we will directly express the modified ompAs in B. fragilis. We performed further optimization of the mutagenesis procedure usingthe full-length B. fragilisompA, including upstream and downstream sequences, as template, for use in a two step recombination gene exchange. We will proceed by expressing and detecting the FLAG-tagged OmpAs directly in theB. fragilisompA deletant(WAL 186), as well as characterizing the phenotypes of B. fragilis withmodified OmpAs.

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Introduction

The dangers of bioterror bacterial or viral agents have been of concern to the military and political leaders for decades. Methods to prevent infection with these agents and to treat infections that may occur are being studied with intensity. Historically, vaccines have been the most efficient method of handling diseases in large populations. Studies of the immune system and vaccine effectiveness have shown that the ideal way to induce a complete immune response of both the mucosal and systemic systems is to administer vaccines in a manner that mimics the natural route of infections. Over the past 15 years, experimental bacterial vaccine vectors have been produced that elicit immune responses against bacterial, viral, protozoan and metazoan pathogens in laboratory animals. Among the advantages of these vaccines is that they are relatively inexpensive to manufacture, they can be given orally, and they can be treated with antibiotics if desired and they effectively induce both humoral and cellular responses. If the organism used as the vector can potentially colonize in the host, the potential of eliciting the appropriate response is increased.

OmpA proteins are among the most conserved of all outer membrane proteins in bacteria; however, the loops expressed on the outer surface are quite variable. Current understanding indicates that the loops are responsible for a variety of virulence characteristics and that they serve as important antigens as well. *Bacteroides fragilis*, most common anaerobic pathogen, is a major component of the stool flora and colonizes the gastrointestinal tract. OmpA is one of the major outer membrane proteins of this organism. Structure/function analysis of the OmpA protein will ultimately identify the exposed loops of the protein and will both elucidate the role of OmpA in the pathogenic process of *Bacteroides fragilis* and will allow us to exploit the unique nature of this abundant outer membrane protein in designing potential vaccine vectors. These vaccines (i.e., *Bacteroides fragilis* with OmpA modified to express specific epitopes) could be designed for a wide variety of infections that might be acquired by ingestion of food or liquid (including potential biodefense related organisms), and the secretory IgA antibodies produced in the gastrointestinal tract could prevent the pathogen or toxin from exiting the GI tract to invade the circulatory system or other organs.

BODY:

We had previously described the OmpA proteins in anaerobes and characterized the *ompA* gene and OmpA protein in *B. fragilis* quite extensively. In earlier work, we purified the OmpA proteins from *B. fragilis* and the closely related organism, *Bacteroides distasonis* (Wexler, 1992). Using reverse genetic techniques (this was prior to the publication of the *B. fragilis* sequence from the Sanger Center), we identified the *B. fragilis ompA* gene. Subsequently, the sequence was confirmed with the data from the Sanger Center, and three additional full length *ompA* homologs were identified both in the ATCC strain, *B. fragilis* 25285, and in the strain more commonly used for molecular manipulations, 638R. The presence of four gene homologs in a single strain suggests that the OmpA protein(s) serves a very important function in the cell. We have previously shown that all four homologs of the *ompA* gene are transcribed, although only OmpA1 is detectable on SDS-PAGE and on Western blot analysis with anti-OmpA antisera. We demonstrated that OmpA is important in maintaining cell structure: 1) the OmpA disruption mutant is shorter and rounder than the parental strain; 2) the OmpA deletant is more sensitive to SDS and to high salt concentrations than the parent strain; 3) *B. fragilis* responds to high salt stress by shutting down expression of both OmpA1 and OmpA4 and assuming a tight, small, round morphology.

Proposed model of *B. fragilis* OmpA and position of four outside loops. Using various bioinformatic tools, we proposed a hypothetical model of the *B. fragilis* OmpA protein which has an eight-stranded β-barrel configuration with four external loops (Fig. 1).

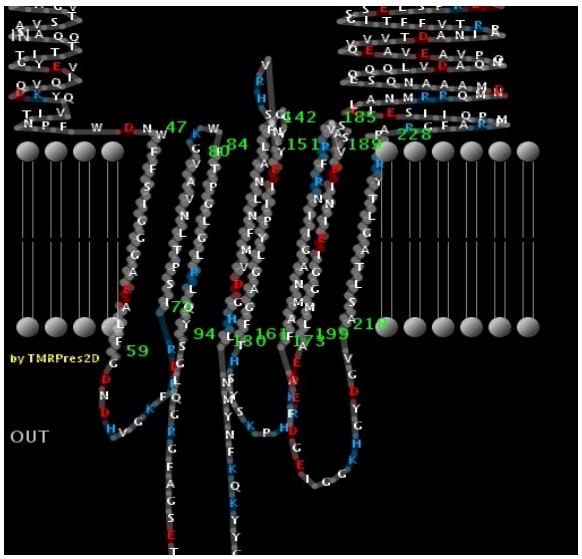

Figure 1: Predicted secondary structure of OmpA showing four exposed loops

This model was used as a basis to decide where to initially place a FLAG tag in a modified *ompA* gene so that the expressed protein would carry an exposed FLAG tag in the outside loops. In case any of the loops confer virulence characteristics to the bacterium, we plan to delete the relevant portion.

Predictions of structural changes with insertions of FLAG (or other tags) or deletions of 5-12 amino acids amino acids to any of the four predicted external loops of OmpA was analyzed using PRED-TMBB (http://biophysics.biol.uoa.gr/PRED-TMBB). This program uses the Hidden Markov Model method to predict and discriminate β-barrel outer-membrane proteins. None of the proposed deletion or insertion significantly affected the predicted topology of OmpA; no other structural changes were seen except for the shortening or lengthening of the external loops. Primer sets (Table 1) were designed to delete nucleotides corresponding to 5-12 amino acids, or insert 24 nucleotides coding for the FLAG tag to *ompA* as shown in Figure 2. Site-directed mutagenesis will be performed to construct the modified OmpAs to study the function of the different loops.

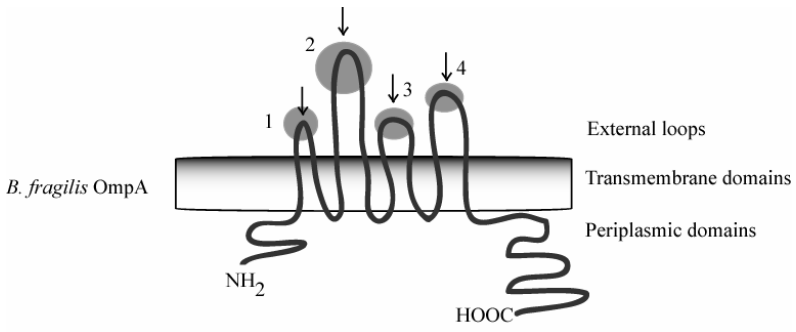

Figure 2: Proposed modifications of OmpA to introduce insertions (arrows) and deletions (shaded circle) to predicted loop domains. The primers for the site-directed mutagenesis are shown in Table 2.

Construction of *ompA* expression vector and initial studies in *E. coli*.

Cloning of mature *ompA* into the pET-27b(+) vector. Our initial objective was to introduce a FLAG tag into gene region coding for one of the outside loops of OmpA and express this modified gene in *E. coli.* The strains and plasmids used in this study are listed in Table 2. The pET-27b(+) plasmid (Novagen) was initially chosen as the vector for these studies.

Site-directed mutagenesis to insert FLAG tags into proposed external loops of OmpA.

We proposed a hypothetical model for *B. fragilis ompA* that is comprised of an eight-stranded β-barrel configuration with four external loops (Wexler, *et al*. 2002 and Figure 1). Based on this model, we used the QuikChange site-directed mutagenesis method (Stratagene) to introduce the FLAG tag to the loops of *ompA*. Briefly, the *ompA* gene was cloned in a supercoiled, dsDNA vector, pET-27b(+). Two synthetic oligonucleotide primers (MWG Biotech) complementary to opposite strands of the vector and containing the desired insert (FLAG tag) were used. Primer pairs ompA_FLAG2_F and ompA_FLAG2_R (Table 1) were designed to insert 24 nucleotides (5'-gat tac aaa gat gat gat gat aaa-3') coding for the FLAG epitope (DYKDDDDK) for display between threonine and alanine residues of the longest loop (Loop 2). PCR amplification (95 ºC for 2 min; 18 cycles of 95 ºC for 1 min, 60 ºC for 1 min and 68 ºC for 7 min; finally, 68 ºC for 10 min) using the high fidelity PfuTurbo DNA polymerase (Stratagene) was performed to generate a mutated plasmid containing staggered nicks. Subsequent incubation with *Dpn*I (specific for methylated and hemimethylated DNA isolated from *E. coli*) was carried out to digest the parental DNA template and select for the synthesized DNA containing the desired insertion. 1-4 μl of the reaction mixture was used to transform *E. coli* competent cells (Top 10 or XL1 strains) and plated onto LB containing appropriate antibiotics for selection (40 μg/mL kanamycin). The position of the FLAG insert was verified by sequencing the purified plasmid (Laguna Scientific).

Recloning of FLAG-ompA into pET 27(b+) with the *pelB* leader. We had previously used pET-27b(+) to overexpress *ompA* for subsequent purification from inclusion bodies (Wexler *et al*. 2002) and for the introduction of the FLAG tags (above). However, in order to export the OmpA protein to the periplasm where it may be incorporated in the outer-membrane, the gene had to be re-cloned in frame with the *pelB* leader sequence in pET-27b(+). The mature FLAG-*ompA* was therefore amplified from the recombinant plasmid using primers ompA_FNcoI and ompA_RBamHI that added *Nco*I and *Bam*HI recognition sites to the 5' and 3' ends, respectively (Table 2). PCR was performed in an Eppendorf Mastercycler Gradient using GoTaq Green primer mix containing GoTaq polymerase, reaction buffer, dNTPs and $MgCl_2$ (Promega). The PCR conditions were as follows: 95 ºC for 2 min; 35 cycles of 95 ºC for 1 min, 60 ºC for 1 min, and 72 ºC for 7 min; and finally 72 ºC for 10 min. The PCR product was purified by agarose gel electrophoresis and gel extraction (Qiagen), and then digested with *Nco*I and *Bam*HI enzymes (New England Biolabs). The resulting fragment was ligated to the vector digested with the same enzymes.

Transformation was performed using *E. coli* Top10 and transformants were selected with kanamycin.

Expression of *FLAG-ompA* in *E. coli.* Although we were able to obtain kanamycin-resistant colonies *E. coli* Top10 cells as described above, these cells did not grow well in LB with 40 ug/ml Kanamycin + 10 mM IPTG, and showed a high tendency to lose the expression plasmid when grown in LB without the antibiotic. We know from other studies that heterologous expression of membrane proteins in *E. coli* may affect cell viability (Shaw and Miroux, 2003), and recombinant plasmids may be lost due to the strong pressure to maintain the membrane integrity. We performed several analyses that indicated that this was the case in our studies. Since these initial studies indicated it may be difficult to achieve stable expression, export to periplasm and integration of *B. fragilis* OmpA into *E. coli* membrane, we decided revise our strategy and instead proceed with expressing the tagged OmpAs directly in the *B. fragilis ompA* deletion strain (186) by a two-step recombination gene exchange protocol. We plan to utilize the first plasmid constructed, i.e. pET-27b(+)::*ompA*-FLAG, for expressing OmpA in inclusion bodies and also to study immune responses to purified modified OmpAs.

Design of plasmid for use in gene exchange protocol in *B. fragilis.* For the gene exchange protocol, we needed to construct a plasmid that contained the upstream and downstream sequences required for recombination as well as the FLAG-*ompA*, and we did not need an expression vector. For that purpose, pBR322 was a more useful cloning vector than pET-27b(+).

Cloning of full-length *ompA* with upstream and downstream sequence. The general scheme for cloning *ompA* is shown in Figure 3. Template DNA was prepared from WAL 108 by suspending the cells in nuclease-free water and heating at 98 ºC for 25 min. The cell suspension was centrifuged for 10 min and the supernatant was used for PCR. To amplify the full-length *ompA* (1.1 kb) including the upstream and downstream sequences (total size 2.7 kb), primers ompAupHindIII and ompAdownRBamHI were used (Table 1). The flanking sequences and restriction sites were added so that we could subsequently reintroduce the *ompA* to the WAL 186 *ompA* deletant strain using the two-step double cross-over technique (Baughn and Malamy, 2002). The PCR conditions were as follows: 95 ºC for 2 min; 5 cycles of 95 ºC for 1 min, 65 ºC for 1 min, 72 ºC for 3 min; 5 cycles of 95 ºC for 1 min, 64 ºC for 1 min, 72 ºC for 3 min; 25 cycles of 95 ºC for 1 min, 63 ºC for 1 min, 72 ºC for 3 min; and finally 72 ºC for 10 min. The PCR product was subjected to agarose gel electrophoresis and purified using Qiagen Gel Extraction kit (Qiagen). The purified product was

digested with *Hind*III and *Bam*HI (New England Biolabs) and ligated into pBR322 that had been similarly digested. The mixture was ligated and used to transform XL1 strains (Stratagene). Transformants were selected in LB supplemented with 10 μg/mL ampicillin. Plasmids were prepared using the Qiagen Miniprep Kit (Qiagen), digested with *Hind*III and *Bam*HI (New England Biolabs) and checked for insert size by agarose gel electrophoresis. Primers corresponding to the gene as well as the upstream and downstream sequences (Table 1) were used to screen transformants, and the sequence on the recombinant plasmid was verified by DNA sequencing (Laguna Scientific).

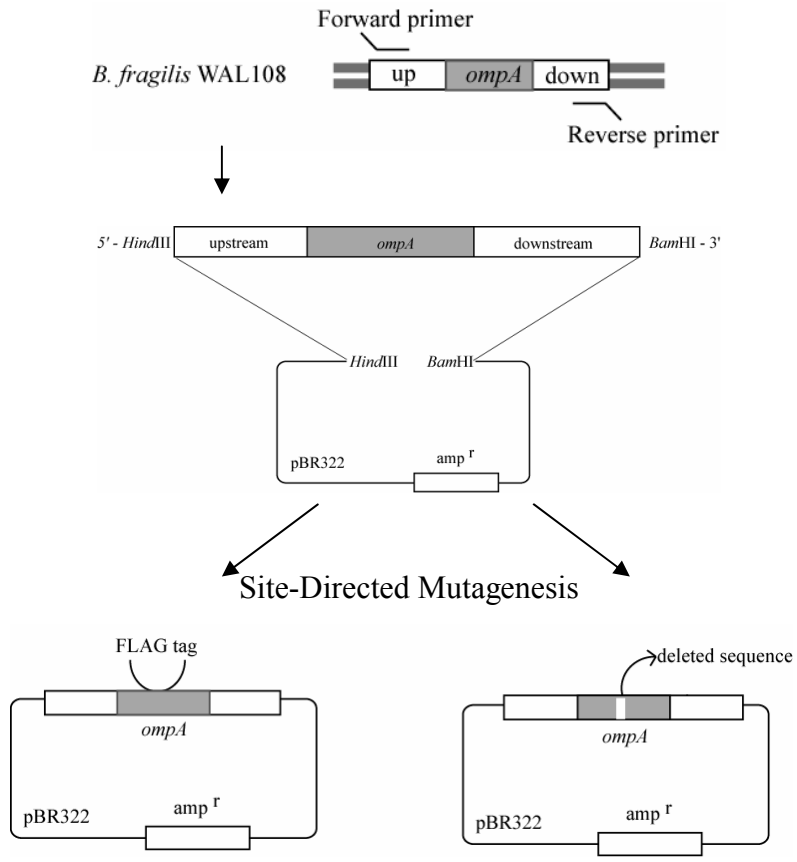

Figure 3: Cloning and site-directed mutagenesis of the full-length *ompA* in the pBR322 vector.

Optimization of the Quickchange protocol for inserting the FLAG tag into pBR322::*upstream-ompA-downstream*. We needed to reoptimize the Quickchange protocol for our application using the *ompA* insert containing the up- and downstream regions. The Quikchange protocol is a simple and broadly applicable method for introducing mutations. However, this mutagenesis system is optimal for primer pairs of 25-45 bases and shorter mutations of less than 5

amino acids in small plasmids. When longer primers are used (e.g., more than 60 bases for our FLAG primers), the efficiency decreases due to primer-dimer formation (Wang and Malcolm, 1999). We performed extensive optimization of the procedure (including testing other suitable plasmid vectors, using a two-stage PCR protocol to circumvent primer-dimer formation, varying the template and primer concentration used during PCR, using Quikchange XL kit with the addition of Quiksolution ^{TM} for large and complex templates, and utilizing competent cells appropriate for larger plasmids).

The plasmid pBR322:: up*ompA*down vector was used as the mutagenesis template to insert the FLAG tag in loops 1, 2 and 3 using the appropriate primer pairs: ompA_FLAG-Loop1F /ompA_FLAG-Loop1R for loop1; ompA_FLAG_2_For / ompA_FLAG_2_Rev for loop 2; and ompA_FLAG-Loop3F / ompA_FLAG-Loop3R for loop 3 (Table 2). The parental plasmid DNA was likewise digested with *Dpn*I, mixed with the amplified *FLAG-ompA*s and the ligation mixture was used to transform XL10 Gold ultracompetent cells. Transformants were selected in LB with 50 μg/mL ampicillin. Initial screening by PCR indicates that the selected transformants have the appropriately sized plasmids. We are currently verifying that these plasmids contain the proper FLAG-tagged OmpA by DNA sequencing.

Once the presence of the FLAG sequence has been confirmed in pBR322::up*ompA*down, the plasmid will be digested with *Bam*HI and *Hind*III, and the *up-ompA-down* fragment will be re-cloned into the pADB242a suicide vector (also digested with *Bam*HI and *Hind*III) using standard molecular biology techniques as outlined in Figure 4. This plasmid will be used to transform chemically competent *E.coli* DH5α cells.

Gene exchange in *B. fragilis*.

A two-step double cross-over technique will be used to perform a gene exchange into *B. fragilis* WAL 186 *ompA* deletant (Baughn and Malamy, 2002) (Figure 4). *E.coli* DH5α cells containing modified pADB242a::up*ompA*down and *E. coli* containing the pK2317 mobilizer plasmid will be mated with the *B. fragilis* WAL 186 *ompA* deletant in a three part mating. The first cross over event (in either the up- or downstream region) will result in a cointegrant containing the suicide vector. The second recombination event will result in either the parental genotype (i.e., *ompA* deletant) or a strain with the modified *FLAG-ompA* inserted between the up and downstream regions. To confirm that the modified loops are exposed in the allele-exchanged *B. fragilis*, the FLAG epitope displayed in OmpA will be detected by Whole-cell ELISA using monoclonal M2 antibody for FLAG and Proteoqwest Kit (Sigma).

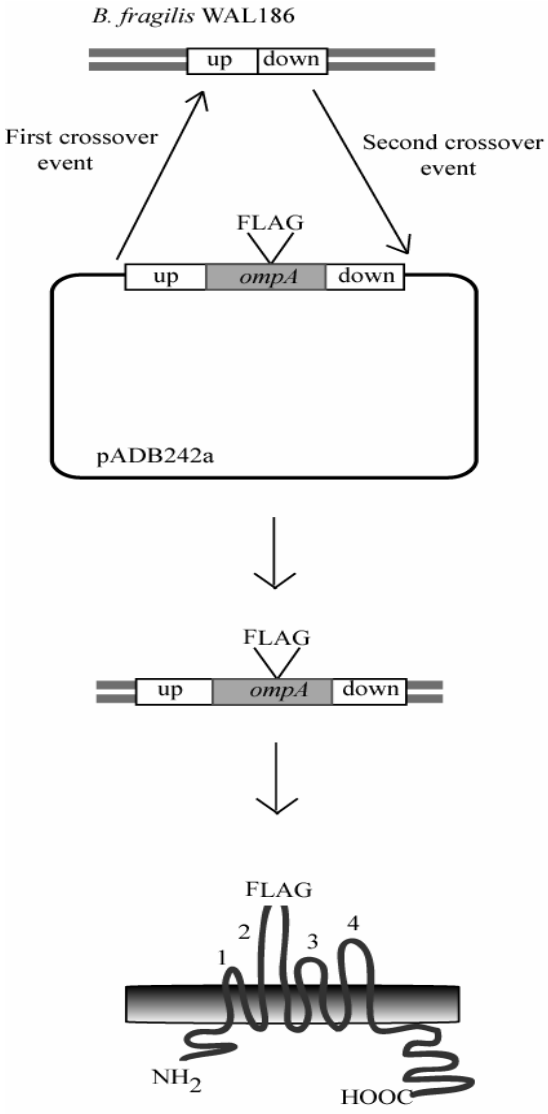

Figure 4: Construction of *B. fragilis* strains expressing modified OmpAs.

Further characterization of the *ompA* deletion mutant. We have previously observed that the WAL 186 *ompA* deletion mutant was more sensitive to SDS and high salt concentration. Furthermore, both the WAL 108 parental strain and the WAL 186 deletant showed altered morphology (small round forms on Gram stain) and reduced expression of *ompA1* and *ompA4* when grown on hyperosmolar media. To investigate the possible role of *B. fragilis* OmpA in the response to various osmotic stresses, we are conducting preliminary studies using phenotype microarray panels (Biolog). The microplate-based assay can be used to monitor cell metabolism in

the presence of osmolytes such as NaCl, KCl, urea, etc. (PM 9 panel) and a pH range of 3.5 to 10 (PM 10 panel). Briefly, WAL 108 and WAL 186 will be grown in BHIS agar anaerobically for 2 days at 37 ºC. Cells will be suspended in the appropriate inoculating fluid at a target cell density and distributed into the wells of the PM panels. The response of cells to the contents of each well will be monitored colorimetrically. Using this approach, we will be able to clarify which responses to specific cell stressors require a functional OmpA.

Effect of OmpA on cytokine induction. To determine whether OmpA can regulate the secretion of cytokines from murine macrophages and dendritic cells, specific ELISAs will be performed using kits for IF-gamma, IL-alpha, IL-10, IL-6, IL-4 and TNF (Biosource). These studies are currently being performed by Dr. Y. Nitzan at Bar Ilan University (Tel Aviv).

Table 1: Primers used in this study

Primers	**Sequence**	**Source or reference**
Primers for inserting *ompA* into cloning and expression vectors		
ompA_FNcoI	5'-CGTTCCATGGAGCAGACTACAATTACGGGA-3'	Tomzynski
ompA_RBamHI	5'-GCGTGGATCCTTATTTAACAGACTCTACTAATA-3'	
ompAupFHindIII	5'- AAGGAAGCTTACAGTCTGGGAGTCGCTACCT-3'	
ompAdownRBamHI	5'-GCTTGATCCTCACTTACCCCATCAGTCAGG-3'	
Primers for inserting the FLAG epitope into *ompA* by site-directed mutagenesis		
ompA_FLAG_2_For	5'-CTTTGCCGGTTCGGAAACAGATTACAAAGATGATGATGATAAAGCTGATTTTGTGAAGAG-3'	This study
ompA_FLAG_2_Rev	5'-CTCTTCACAAAATCAGCTTTATCATCATCATCTTTGTAATCTGTTTCCGAACCGGCAAAG-3'	
ompA_FLAG-Loop1F	5'-AAGCACTGTTTGGCGATAATGACCATGATTACAAAGATGATGATGATAAAGTCGGGAAATTCAGGGA-3'	

ompA_FLAG-Loop1R	5'-TCCCTGAATTTCCCGACTTTATCATCATCATCTTTGTAATCATGGTCATTATCGCCAAACAGTGCTT-3'	
ompA_FLAG-Loop3F	5'-GAGCCGGTTTTACTCATTCATATTCGAAAGATTACAAAGATGATGATGATAAACCTCATCGTGAGGCG-3'	
ompA_FLAG-Loop3R	5'-CGCCTCACGATGAGGTTTATCATCATCATCTTTGTAATCTTTCGAATATGAATGAGTAAAACCGGCTC-3'	
ompA_FLAG-Loop4F	5'-GGCCGAAGATAAGTTTGACGGAGATTACAAAGATGATGATGATAAAGAAATTGGCGGAAAACATGGAT-3'	
ompA_FLAG-Loop4R	5'-ATCCATGTTTTCCGCCAATTTCTTTATCATCATCATCTTTGTAATCTCCGTCAAACTTATCTTCGGCC-3'	
Primersfor deleting *ompA* loop segments by site-directed mutagenesis		
ompA_del_Floop1	5'-GCACTGTTCGGCGATGACCGTATCAGTCC-3'	This study
ompA_del_Rloop1	5'-GGACTGATACGGTCATCGCCGAACAGTGC-3'	
ompA_del_Floop2	5'-GCAGTACAGTGGCATGAATCTTCACGGG-3'	
ompA_del_Rloop2	5'-CCCGTGAAGATTCATGCCACTGTACTGC-3'	
ompA_del_Floop3	5'-CCGGTTTTACTCATCGTGAGGCGTTTGC-3'	
ompA_del_Rloop3	5'-GCAAACGCCTCACGATGAGTAAAACCGG-3'	
ompA_del_Floop4	5'-CCGAAGATAAGTTTGACTATGACGGTGTTGC-3'	
ompA_del_Rloop4	5'-GCAACACCGTCATAGTCAAACTTATCTTCGG-3'	
Primers for PCR confirmation and sequence analysis		
ompA_FLAGinsert	5'-GATTACAAGGATGATGATGATAA-3'	This study
ompA_loop2_For	5'-GGCGATAATGACCATGTC-3'	
ompA_intF255	5'-GCTATGAACGCAGGTATTATCAA-3'	
ompA_intR256	5'-TAGCATCCGTAACGACTACCTGT-3'	
ompA_delJunF257	5'-GGAAAGATTGATTATGCCGTTAC-3'	
ompA_delJunR258	5'-GAGAAAAGGCTGTGAAGTAGCAA-3'	

The underlined nucleotides code for the FLAG tag.

Table 2: Strains and plasmids used in this study

	Description or relevant marker	**Source or reference**
Strains		
WAL 108	*B. fragilis* ADB77; TM400, ΔthyA, rifampicin^{R}	
WAL 186	*B. fragilis* ADB77 ΔompA	
	E.coli Top 10, host strain for cloning	
	E.coli Top 10/ pET-27b(+)::ompA; kan ^{R}	Stratagene
	E.coli Top 10/ pET-27b(+)::ompA FLAG; kan ^{R}	This study
	E.coli XL-1 Blue, host strain for cloning	Stratagene
	E.coli XL-1 Blue/pBR322; tet ^{R} amp ^{R}	This study
	E.coli XL-1 Blue/ pBR322::upompAdown; amp ^{R}	This study
	E.coli XL-10-Gold, Ultracompetent cell	Stratagene
	*E. coli/*pK2317	Malamy
Plasmids		
	pET-27b(+); kan ^{R}, expression vector	Tomzynski
	pET-27b(+)::*ompA*; kan ^{R}	This study
	pET-27b(+)::*ompA* FLAG; kan ^{R}	
	pBR322; tet ^{R} amp ^{R} , cloning vector	
	pBR322::up*ompA*down; amp ^{R}	
	pADB242a *thyA*; tet ^{R}	Malamy

KEY RESEARCH ACCOMPLISHMENTS:

- Design of primers for introducing FLAG tags to four predicted external loops of OmpA and for deleting segments of OmpA to the used in the phenotypic analysis of *B. fragilis* with modified OmpAs

- Optimization of protocols for inserting FLAG tags to selected external loops of OmpA using Quikchange site-directed mutagenesis kit and construction of FLAG insertion mutants in loops 1, 2 and 3.

- Initial studies of cloning and expression of mature *ompA* in *E. coli* using the *pelB* leader sequence of pET-27b(+) vector.

- Cloning of full-length *B. fragilis ompA* gene including upstream and downstream sequences for site –directed mutagenesis and subsequent recombination to an *ompA* deletant strain using the pBR322 vector

- Cytokine assays are currently being performed using over-expressed *ompA* as well as OmpA fragments

REPORTABLE OUTCOMES:

H.M. Wexler, 2005. The function of the OmpA outer membrane protein in *Bacteroides fragilis*. Abstracts. American Society for Microbiology General Meeting, Atlanta, GA.

H.M. Wexler. 2006. Bacteroides fragilis OmpA: Utility as a live vaccine vector for biodefense agents: Construction of an ompA deletant and characterization of the function of OMPA . DoD MHRF, May 1-4, 2006, Puerto Rico.

H.M.Wexler, L. Pumbwe, E.K. Read, and T.J. Tomzynski and E. Tenorio. 2007. Function of the OmpA outer membrane protein in *Bacteroides fragilis*. Submitted for publication.

CONCLUSIONS

We are developing a novel antigen presentation system in which a variety of epitopes can be inserted into the OmpA protein and presented by *B. fragilis*. A hypothetical model of *ompA* showed an eight-stranded β-barrel configuration with four external loops which could be modified for eventual use in a vaccine vector. We used a PCR-based site-directed mutagenesis technique to insert the FLAG marker into recombinant *ompA* cloned in the pET-27b(+) expression system. Initial studies indicated difficulty in achieving stable expression, export to periplasm and integration of *B. fragilis* OmpA into *E. coli* membrane. To overcome this, we instead cloned in pBR322 vector the full-length *B. fragilis ompA* gene, including upstream and downstream sequences required for subsequent recombination to an *ompA* deletion strain. We designed primers (FLAG insertion or amino acid deletions) for modifying the predicted loops, and performed further optimization of the mutagenesis procedure using this new template. Once the mutations have been verified by sequencing, we will proceed with expressing the tagged OmpAs directly in *B. fragilis* WAL 186 *ompA* deletion strain. We are also investigating the role of OmpA and its various loops in cytokine induction as well as the cellular response to osmolytes and pH changes.

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APPENDICES: All figures and tables are embedded in the text.

SUPPORTING DATA: All figures and tables are embedded in the text.